

# **THREE PHASE ENZYMATIC REACTOR FOR CONTINUOUS OPTICAL RESOLUTION**

*A thesis submitted in Partial Fulfillment  
of the Requirements for the  
Degree of Master of Technology*

*by*

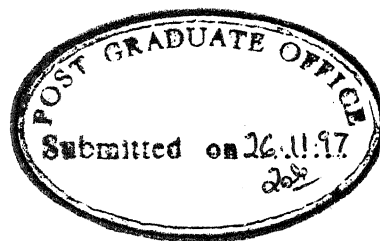
**Cini Padmanabhan**

*to the*

**Department of Chemical Engineering  
Indian Institute of Technology, Kanpur**

*November 1997*

# CERTIFICATE



It is certified that the work contained in the thesis entitled **Three Phase Enzymatic Reactor for Continuous Optical Resolution** by **Cini Padmanabhan** has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

*P. Wangikar*  
26/11/97

Dr. Pramod Wangikar  
Assistant Professor  
Department of Chemical Engineering  
Indian Institute of Technology, Kanpur.

November 26, 1997

15 DEC '997  
CENTRAL LIBRARY  
I. I. T., KANPUR

Vol. No. A 124454



A124454

CHE-1997-D-PAD-THR

# ACKNOWLEDGEMENTS

I am extremely grateful to my advisor, Dr. Pramod Wangikar for his guidance and patience throughout my M.Tech project. Without his help and encouragement this work would not have been possible. My sincere thanks to Dr. Y. D. Vankar, Department of Chemistry for generously permitting me to use all the facilities of his laboratory and also for the valuable suggestions and counselling he has given during every phase of my thesis work.

I am thankful to Sangeeta , who by her warmth and liveliness made the tedious lab work a pleasant experience for me. I also thank Shahi, Kumar, Pachaymuthu , and Anuradha of Core Lab 205E for their friendliness and willingness to lend me a helping hand anytime I wanted.

My special thanks goes to Dr. Joseph John of EE Department for kindly permitting me to use his PC and also to my friends Balu, Imran, Rani, Sushmita and Brishti , without whose help the completion of this work in time would have been impossible.

The MHRD scholarship is gratefully acknowledged for the assistantship provided during my M. Tech studentship.

I am thankful to all my friends at IITK who have made my stay here a pleasant and memorable one. I would like to thank my parents and brother for their love, support and encouragement. Finally, a word of thanks to my beloved uncle and aunt who have always been there for me.

## ABSTRACT

Optically pure compounds find wide applications as pharmaceuticals, food additives, agrochemicals etc. A novel "three phase enzymatic reactor" was devised for continuous optical resolution of racemic mixtures of alcohols. The reactor was based on the principle of enzyme mediated enantioselective transformation followed by extraction at two liquid-liquid interfaces. The three phases of the reactor consisted of two aqueous phases, physically separated from each other, and a third organic phase in contact with both the aqueous phases. Lipases were used for enzymatic reactions. The performance of the reactor was evaluated on a laboratory scale. Percentage of transport and enantiomeric excesses were calculated. No passive transport was detected in any of the runs, and the rate of facilitated transport depended on the substrate and enzyme used. The laboratory scale experiments demonstrated the effectiveness of the reactor to carry out optical resolution. Scaling up of the "three phase reactor" can be done after further studies on the overall kinetics and selection of appropriate enzyme- solvent system, for industrial applications.

# CONTENTS

## List of Figures

## List of Tables

## Chapter

<b>1</b>	<b>Introduction</b>	<b>1</b>
1.1	Enantiomerism and Importance of Enantiopure Compounds	2
1.2	Resolution of Optically Active Compounds	4
1.2.1	Methods of Resolution	4
1.3	Enzymes as Synthetic Tools	5
1.4	Why are Lipases Interesting Catalysts	7
1.5	Asymmetric (Trans)Esterifications Catalysed by Lipases	8
1.5.1	Resolution of Racemic Alcohols	9
1.5.2	Optically Active Carboxylic Acids	11
1.6	Applications of Lipases	12
1.7	Determination of Enantiomeric Purity	14
1.8	The Proposed Work: Three Phase Enzymatic Reactor	17
<b>2</b>	<b>Experimental Section</b>	<b>18</b>
2.1	General	18

2.2	Materials	19
2.2.1	Preparation of Pig Liver Acetone Powder(PLAP)	19
2.2.2	Preparation of Phosphate Buffer	19
2.2.3	Substrates	20
2.2.4	Esterification of Lactic Acid	20
2.2.5	Preparation of 1,2-Propane diol	20
2.2.6	Esterification of Mandelic Acid	21
2.2.7	Preparation of 1-phenyl, 1,2-ethane diol	21
2.3	Biphasic Reactions	22
2.3.1	Reaction using PPL	22
2.3.2	Reaction using PLAP	22
2.4	Three Phase Reactions	22
2.4.1	General Procedure for Three Phase Reactions	24
2.4.2	Control Reaction	24
3	<b>Results and Discussion</b>	26
3.1	Objectives	26
3.2	Selection of Enzyme	27
3.3	Selection of Substrates	28
3.4	Selection of the Phases	29

3.5	Biphasic Reactions	29
3.6	Three Phase Reactions	30
3.6.1	Run 1: Lactic acid + Vinyl Acetate Using PPL in aq(1) and PPL in aq(2)	30
3.6.2	Run 2: Lactic acid + Vinyl Acetate Using PPL in aq(1) and PLAPin aq(2)	33
3.6.3	Run 3 : 1,2-Propane diol + Vinyl Acetate Using PPL in aq(1) and PPL in aq(2)	36
3.6.4	Run 4: 1-Phenyl, 1,2-ethane diol + Vinyl Acetate Using PPL in aq(1) and PPL in aq(2)	39
3.6.5	Control Reactions	41
3.6.6	Overall Results of the Three Phase Reactions	42
3.7	Future Work: Overall Kinetics of the Three Phase Enzymatic Reactor	43
4	Conclusions	46
	References	48



# LIST OF FIGURES

Fig. 1	Schematic of Three Phase Enzymatic Reactor.	23
Fig. 2	Schematic of Three Phase Reactor Showing Reactions at the Interface.	27
Fig. 3	Rotation vs Time of Three Phase Reaction Run 1- Lactic Acid + Vinyl Acetate using PPL in aq(1) and PPL in aq(2).	32
Fig. 4	Rotation vs Time of Three Phase Reaction Run 2- Lactic Acid + Vinyl Acetate using PPL in aq(1) and PLAP in aq(2).	35
Fig. 5	Rotation vs Time of Three Phase Reaction Run 3- 1,2-Propane Diol using PPL in aq(1) and PPL in aq(2).	38
Fig. 6	Rotation vs Time of Three Phase Reaction Run 4- 1-Phenyl, 1,2-Ethane Diol using PPL in aq(1) and PPL in aq(2).	40
Fig. 7	Expected Plot for Kinetics of Three Phase Enzymatic Reactor.	45

# LIST OF TABLES

Table 1	Overview of Commonly used Commercially Available Lipases	8
Table 2	Results of Biphasic Reactions using Lactic Acid	29
Table 3	Rotation vs Time of Three Phase Reaction Run 1- Lactic Acid + Vinyl Acetate using PPL in aq(1) and PPL in aq(2).	31
Table 4	Rotation vs Time of Three Phase Reaction Run 2- Lactic Acid + Vinyl Acetate using PPL in aq(1) and PLAP in aq(2).	34
Table 5	Rotation vs Time of Three Phase Reaction Run 3- 1,2-Propane Diol using PPL in aq(1) and PPL in aq(2).	37
Table 6	Rotation vs Time of Three Phase Reaction Run 4- 1-Phenyl, 1,2-Ethane Diol using PPL in aq(1) and PPL in aq(2).	39
Table 7	Overall Results of the Three Phase Reactions.	42

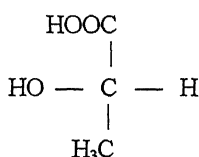
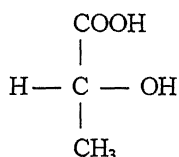
# CHAPTER 1: INTRODUCTION

The synthesis of optically active compounds has been a challenging area of organic synthesis with a long history. In recent years, interest in the synthesis of optically active compounds in homochiral form has gained new impetus as a consequence of ever increasing awareness about the importance of optical purity in the context of biological activity. An increasing interest in understanding biological processes and the general recognition that chirality plays a crucial role in nature fostered a tremendous effort in enantioselective synthesis. In the course of synthesising natural products and designing new target compounds, the fact that enantiopurity is related to biological properties, is acknowledged (Schoffers et al, 1996). Several biologically active molecules, such as pharmaceuticals, food additives, agrochemicals etc. are often chiral molecules. Research over the past twenty years has resulted in great advances in developing efficient and economic methods for the synthesis of a variety of optically active compounds (de Zoete et al, 1994). Consequently, enantioselective synthesis has become a major branch of organic chemistry. The biocatalytic approach, in particular, has recently emerged as a major tool for the synthesis of homochiral compounds with enzymes being increasingly recognised as potential chiral catalysts.

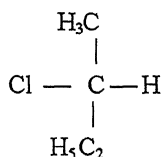
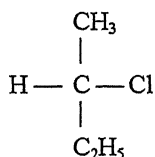
## 1.1. ENANTIOMERISM AND IMPORTANCE OF ENANTIOPURE COMPOUNDS

Carbon atoms carrying four different substituents possess a unique property (Koskinen, 1993). The substituents can be arranged in two alternative ways to bring about two forms of the molecule with the same constitution. The two forms of the molecule are related to each other as non superimposable mirror images. Such mirror image isomers are called enantiomers.

eg.(a)Lactic acid.



(b)Sec-butyl chloride



Enantiomers have identical physical properties, except for the direction of rotation of the plane of polarised light (optical activity). They also have identical chemical properties, except toward optically active reagents. The non superimposability of mirror images that brings about the existence of enantiomers, also gives them their optical activity. Hence enantiomers are also referred to as optical isomers.

Enantiomers interact differently within an organism and can display various activities. Despite their close similarity, one isomer of a pair of enantiomers may serve as a nourishing food, or as an antibiotic, or as a powerful heart stimulant, and the other isomer may be either neutral or harmful. Some property differences between enantiomers are enormous, ranging from distinguishable smells and flavours to teratogenic effects. Among naturally occurring enantiomers, lemons and oranges both contain limonene, the different enantiomers giving rise to subtle changes in aroma properties (Koskinen, 1993). The sugar (+)-glucose plays a unique role in animal metabolism and is the basis of fermentation industry, yet (-)-glucose is neither metabolized by animals nor fermented by yeast (Morrison and Boyd, 1995). The hormonal activity of (-)-adrenaline is many times that of its enantiomer. Among amino acids, only one asparagine and one leucine are sweet, and only one glutamic acid enhances the flavour of food.

In the drug industry, enantiopure products are playing an increasingly important role. For example, only one stereoisomer of chloromycetin is an antibiotic. (+)-Ephedrine not only has no activity as a drug, but it interferes with the action of its enantiomer. The (S)-enantiomer of the drug propranolol, which is used to treat hypertension, has hundred times of  $\beta$ -adrenergic activity of the (R)-enantiomer. It is worth mentioning the infamous thalidomide case of the 1950s (Schoffers et al, 1996), in this context. Between 1958 and 1962, the worldwide use of the racemic drug thalidomide by pregnant women resulted in severe birth defects in approximately 10,000 children. Later research revealed that while the (R)-enantiomer of thalidomide is an effective sedative, the (S)-enantiomer is

teratogenic. Events like this have stimulated drug regulation legislation including highly restrictive guidelines for the marketing of synthetic chiral drugs. There is a strong emphasis on the development of single stereoisomers for the marketing of new drugs.

## **1.2. RESOLUTION OF OPTICALLY ACTIVE COMPOUNDS**

Optically active compounds generally exist as a fifty-fifty mixture of each enantiomer, which is called the racemic mixture. This mixture is optically inactive. Ordinary methods like fractional crystallization, fractional distillation, chromatography etc. fail to resolve a racemic mixture due to lack of difference in physical properties of the enantiomers..

### **1.2.1. Methods of Resolution**

Resolution can sometimes be achieved by direct crystallization, although this is mainly used for improving the optical purity of many crystalline compounds (Koskinen,1993) Chromatography using a large number of chiral columns can be used to achieve resolution. There is another indirect method by which the compound to be resolved is derivatized with a suitable chiral reagent, and the derivatizing agent is again cleaved after chromatographic separation. Asymmetric transformations could provide an interesting method for obtaining enantiopure compounds. One can resort to a variety of methods to accomplish the synthesis of optically pure compounds, and among these, the application of enzymes has become widely accepted. By far, the best asymmetric syntheses are achieved in nature by using enzymes as catalysts.

### 1.3. ENZYMES AS SYNTHETIC TOOLS

Enzymes have become highly attractive catalysts having valuable industrial and medical applications. For almost every type of chemical reaction there exists an enzyme catalyzed equivalent (Schoffers et al, 1996). A variety of reactions can be mediated by using enzymes, such as oxidation, reductions, hydrolyses, condensations and isomerizations. Enzyme catalyzed reactions are superior to conventional chemical methods due to several factors (Gandhi et al, 1995). They catalyse all biological reactions in vivo. They also catalyze reactions involving both natural and unnatural substrates in vitro. Enzymes offer many advantages over conventional catalysts as listed below (Wong and Whitesides, 1994).

1. Enzymes accelerate the rate of reactions, and operate under mild conditions.
2. Enzymes are highly selective.
3. Enzymes may be subject to regulation.
4. Enzymes are relatively unstable.
5. Enzymes are chiral, and can show enantiodifferentiation.

Enzymes have become extremely important in the preparation of enantiomerically pure unnatural compounds (Schoffers et al; 1996). The regioselective, or position selective modification of polyfunctional compounds has been possible by enzyme catalyzed reactions and has found many synthetic applications (Klibanov et al; 1994). Enzymes are

chiral molecules and their ability to discriminate between enantiomers of a racemic substrate is of well recognized preparative value. The resolution of racemic mandelate by pig liver esterase (PLE) was published by Dakin as early as 1903.  $\alpha$ -chymotrypsin from bovine pancreas was one of the earliest enzymes to be investigated preparatively and has been widely used for the resolution of amino acids.

The native enzymes can be modified to suit the requirements via immobilization, chemical modification of active site and site directed mutagenesis that may result in increased operational stability and change in stereoselectivity. The past fifteen years have seen dramatic changes in the use of enzymes due to new developments in chemistry and biology and new requirements in industry (Wong and Whitesides, 1994). Large number of enzymatic reactions have been demonstrated to transform natural or unnatural substrates stereoselectively to synthetically useful intermediates or final products. To scale up enzymatic reactions, new techniques have been developed to improve the stability of enzymes and to facilitate their recovery for reuse. New enzymes have been discovered that are key elements of molecular genetics and recombinant DNA technology. Recombinant DNA technology has made possible, in principle, the low cost production of proteins and enzymes and the rational alteration of their properties. With the advent of monoclonal antibodies, it has become possible to synthesize artificial enzymes - catalytic antibodies - that have been shown to catalyze required reactions in stereoselective manner.

The Enzyme Commission has classified enzymes in six main groups according to the type of reaction they catalyze.



1. Oxidoreductase
2. Transferase
3. Hydrolase
4. Lyase
5. Isomerase
6. Ligase

Enzymes from group (1) through (4), especially hydrolytic enzymes, have found broad application in organic transformations.

#### **1.4. WHY ARE LIPASES INTERESTING CATALYSTS?**

Lipases belong to the class of serine hydrolases and they occur widely in nature. The natural function of lipases is to catalyze the hydrolysis of triglycerides to the corresponding fatty acids and glycerol. They are also found to catalyze the reverse reaction of synthesis of esters just as efficiently. A large variety of lipases, from different sources are commercially available at a relatively low cost. Though all of them are called lipases and catalyze the same reactions, their specificities are distinct. Another beneficial feature of lipases is their excellent stability (de Zoete et al, 1994). They are robust enzymes that can tolerate relatively hostile conditions like organic solvents and elevated temperatures. CA lipase, for example, functions at 80°C in hydrophobic solvents. Lipases are ecologically beneficial natural catalysts. All these features have made lipases widely acceptable as catalysts for synthesis of optically pure pharmaceuticals, agrochemicals, flavours and food additives etc.

**Table 1:** Overview of commonly used commercially available lipases

Lipase	Supplier	Trade Name
C rugosa	Sigma	
	Amano	Amano R, Amano AY
C Antarctica	Novo Nordisk A/S	Novozyme 435
Rh miehei	Novo Nordisk A/S	
Humicola	Novo Nordisk A/S	
PPL	Sigma	
Pancreatin	Sigma	
Pseudomonas	Amano	Amano PS Amano AK
Rhizopus arrhizus	Boehringer Mannheim	

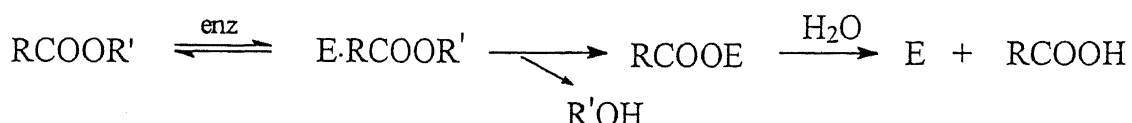
## **1.5. ASYMMETRIC (TRANS)ESTERIFICATIONS CATALYZED BY LIPASES**

The ability of lipases and proteases to catalyze the asymmetric hydrolysis of chiral esters in water has been profitably exploited for years (Klibanov, 1990). Chiral resolution of racemic alcohol or acid is carried out by first chemically esterifying it and then by lipase

catalysed enantioselective hydrolysis. If the lipase reacts with only one enantiomer of the ester, then once a fifty percent conversion is achieved, this enantiomer is completely hydrolyzed to the alcohol and acid, while the unreacted enantiomer remains in the ester form. Even if the enantiodiscrimination is not absolute, a partial resolution is still achieved, with the optical purity dependent on the degree of conversion.

The general mechanism of such reactions can be depicted as shown in scheme 1.

**Scheme1:**



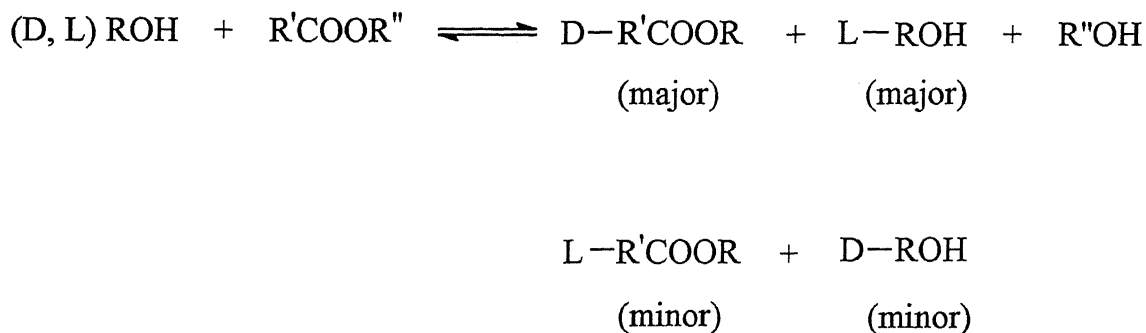
where E is the lipase, RCOOR' is the hydrolyzable ester, E-COOR' is the non-covalent enzyme - substrate complex (the Michaelis complex) and RCOOE is the covalent acyl-enzyme intermediate. The acyl-enzyme intermediate is formed via the acylation of the active center's nucleophile by the acyl moiety of the substrate ester. In water, the acyl-enzyme is subsequently hydrolyzed, thereby regenerating the free enzyme and producing the acid. In principle, other nucleophiles may compete with water for RCOOE, but in aqueous solutions, hydrolysis prevails.

### 1.5.1. Resolution of Racemic Alcohols

A racemic alcohol can be resolved via an enzymatic esterification or transesterification. In this case, the alcohol does not need to be esterified prior to the enzymatic reaction, thereby saving an extra step.

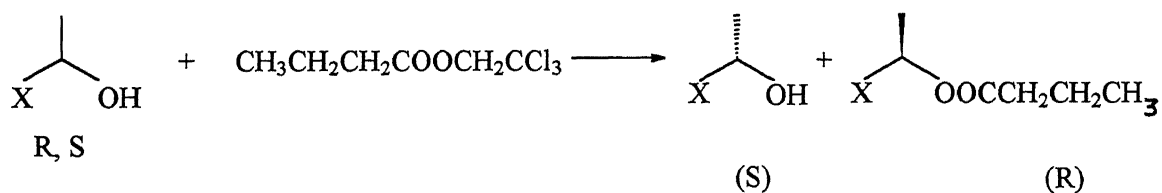
As shown in Scheme 2, if the D-isomer is a better substrate than the L-isomer for the enzyme, accumulation of the D-ester and the unreacted L alcohol will be observed. In the reverse reaction, however, the D-ester is a better substrate converted to D alcohol. The enantiomeric excesses of both D-ester and L alcohol will therefore decrease progressively as the extent of the reverse reaction increases (Wong et al, 1988)

**Scheme 2:**



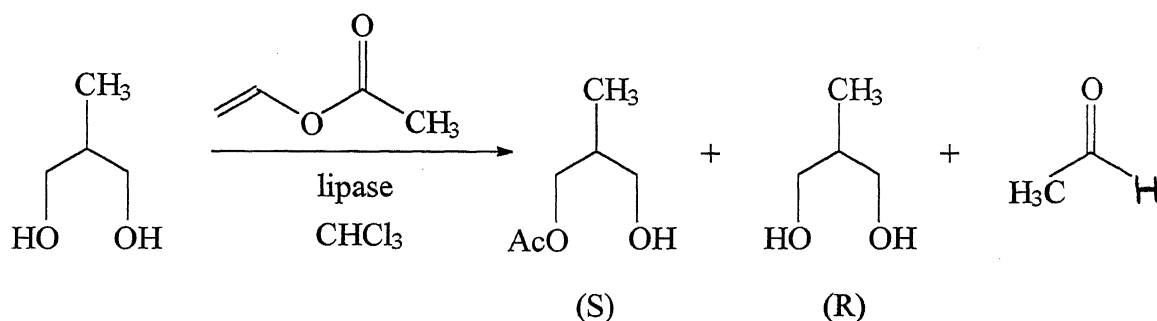
Several racemic secondary alcohols were resolved in anhydrous ether or heptane by using crude porcine pancreatic lipase (PPL) as shown in scheme 3 (Klibanov, 1990).

**Scheme 3:**



where X= hexyl decyl, tetradecyl or phenyl. The S alcohols and R esters, formed enzymatically and separated by conventional means, were found to have enantiomeric excess (ee) in the range 90 - 100 %.

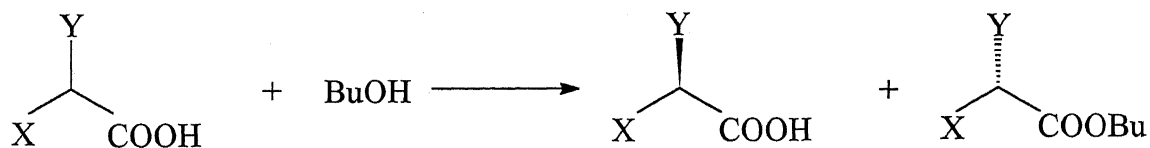
Another convenient way to make an enzymatic transesterification essentially irreversible is to employ enol esters as the acylating agent. The leaving groups released from such esters tautomerize to aldehydes or ketones, thus shifting the equilibrium of the reaction to the right side.



### 1.5.2. Optically Active Carboxylic Acids

Asymmetric esterifications and transesterifications catalyzed by lipases can also be used for the resolution of racemic acids. It has been reported in literature of the stereoselective esterification of racemic acids in hexane using lipase. (Scheme 4)

**Scheme 4:**



where X= Br, Cl

Y=  $\text{CH}_3$ ,  $\text{CH}_3(\text{CH}_2)_3$ , Ph.

High optical purities were obtained in many cases. The same transformations can be conducted in a less efficient manner in biphasic aqueous - organic mixtures.

Lipases have been characterised by their increased activity when acting at lipid -water interface of a micellar or emulsified substrate. This change in activity is called interfacial activation, but is not a general phenomenon (Uppenberg et al, 1995). Lipases retain their activity and enantioselectivity in organic solvents as well as aqueous media (Chen et al, 1989).

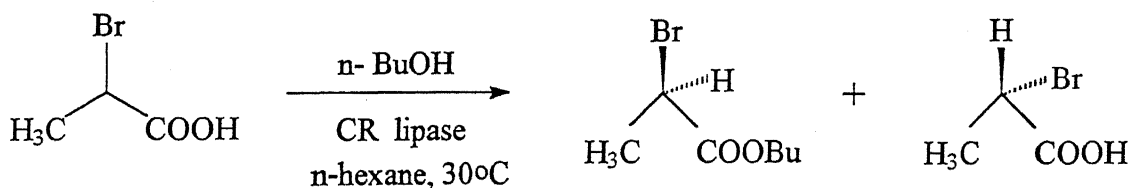
## 1.6. APPLICATIONS OF LIPASES

Lipases have been widely applied in recent years in the commercial synthesis of optically pure pharmaceuticals, agrochemicals, flavours and food additives. Commercially useful lipase catalyzed transformations includes synthesis of intermediates in the manufacture of herbicides, beta- blockers and antihypertensive drugs, laundry detergents etc.

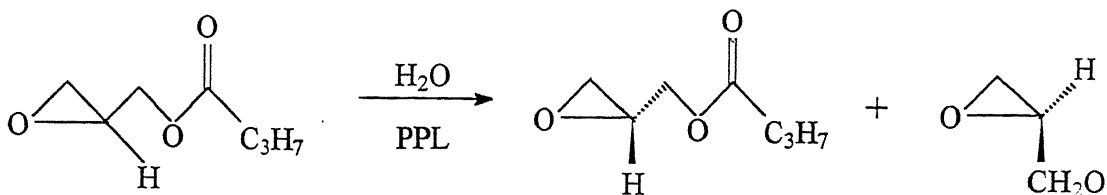
Some examples of lipase catalysed chiral resolutions are shown in scheme 5.(de Zoete et al, 1994).

### Scheme 5:

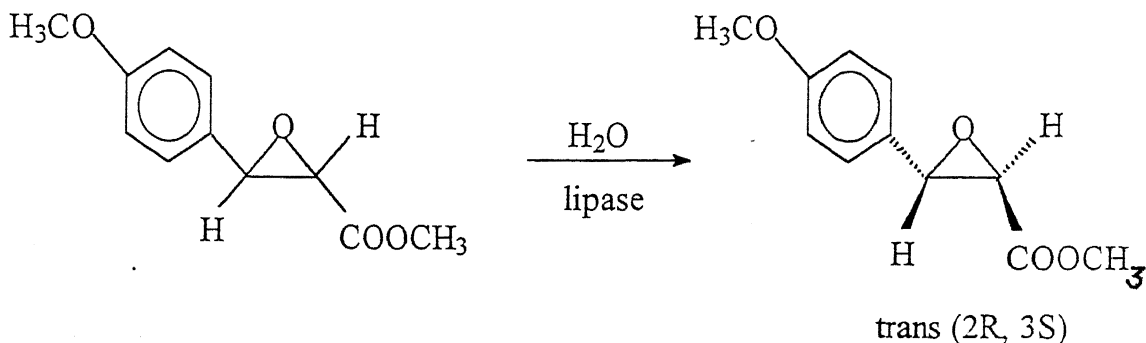
#### Reaction 1



## Reaction 2



## Reaction 3



The resolution of 2-bromo propionic acid (Reaction 1) gives an intermediate for the synthesis of optically pure (R)- $\alpha$ -phenoxy propionic acid herbicides. This process has been commercialised by Chemie Linz in Austria. The resolution of glycidyl butyrate (Reaction 2) and p-methoxy phenyl glycidate ester (Reaction 3) gives intermediates in the preparation of optically active  $\beta$ -blockers and anti hypertensive drug, Diltiazem respectively. These have been commercialised by DSM-Andeno.

Lipase catalyzed reactions have been utilized in the synthesis of selected types of biologically active compounds (Theil, 1995). Prostaglandins, prostacyclins, thromboxanes and their synthetic analogs play an important role as bioregulators in human and animal organisms. Lipases of different origin have been successfully applied on the synthesis of

enantiomerically pure building blocks for prostaglandins and related compounds. Chemoenzymatic approaches including the use of lipases are among the favoured methods to prepare enantiomerically pure nucleoside analogs starting from various types of prochiral or racemic building blocks. There is an increasing interest in the synthesis of nucleoside analogs due to their antitumor and antiviral properties including their activity against the human immunodeficiency virus (HIV). Biocatalytic lipase mediated enantioselective steps are made use of in the manufacture of antibiotics, alkaloids, terpenoids etc. It can be expected that lipase catalyzed reactions will play an increasingly important role in the preparation of non racemic chiral biologically active compounds in the laboratory scale as well as in the industrial production.

## 1.7. DETERMINATION OF ENANTIOMERIC PURITY

According to Koskinen (1993), two measures of enantiomeric purity are in common use; optical purity, based on the optical rotation of the compound, and enantiomeric excess, which is independent of the optical behaviour of the compound.

The optical purity (% op) is defined as the ratio between the observed specific rotation

$[\alpha]_{\text{obs}}$  and the maximum specific rotation  $[\alpha]_{\text{max}}$ .

$$\% \text{ op} = \frac{[\alpha]_{\text{obs}}}{[\alpha]_{\text{max}}} \times 100$$

The specific optical rotation  $[\alpha]$  measured using a polarimeter which gives the rotation angle  $\alpha$  (in degrees) of plane polarised light of wavelength  $\lambda$  (usually the sodium D-



line, 598 nm, is used) in a cell typically 1 dm long in a sample concentration  $c$  (given in gms/100ml).

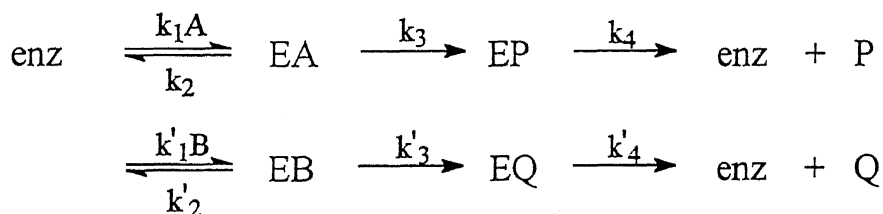
$$[\alpha] = \frac{\alpha}{lc}$$

Enantiomeric excess(%ee) is defined as the excess of one enantiomer over the other.

$$\% ee = \frac{|\% R - \% S|}{|\% R + \% S|} \times 100$$

Equations and useful graphs for the quantitative treatment of biochemical kinetic resolution data have been developed (Chen et al, 1982). These expressions have been verified experimentally, and they possess predictive values in relating the parameters of the extent of conversion of racemic substrate( $c$ ), the optical purity expressed as enantiomeric excess( $ee$ ) and the enantiomeric ratio( $E$ ).

If A and B are the fast and slow reacting enantiomers that compete for the same site on the enzyme. For a simple 3 step kinetic mechanism assuming the reaction is virtually irreversible and there is no product inhibition:



The ratio of the two partial reaction rates( $v_A$  and  $v_B$ ) is shown by steady state kinetics to be

$$\frac{v_A}{v_B} = \frac{V_A}{V_B} \frac{K_B}{K_A} \frac{A}{B}$$

where  $V_A$ ,  $K_A$  and  $V_B$ ,  $K_B$  denote maximal velocities and Michaelis constants of the fast and slow reacting enantiomers, respectively.

The discrimination between two competing enantiomers (A & B) by enzymes is dictated by the enantiomeric ratio  $E$ , which is also the ratio of the specificity constants,  $V/K$ .

$$\frac{\ln(A/A_0)}{\ln(B/B_0)} = \frac{V_A/K_A}{V_B/K_B} = E$$

In instances where kinetic resolution experiments are conducted by the selective destruction of one of the antipodes, the relation between the extent of conversion ( $c$ ) and the enantiomeric excess of the recovered substrate fraction ( $ee(s)$ ) for various values of  $E$  is governed by

$$\frac{\ln[(1 - c)(1 - ee(S))]}{\ln[(1 - c)(1 + ee(S))]} = E$$

where

$$c = 1 - \frac{A + B}{A_0 + B_0}$$

$$ee(S) = \frac{B - A}{A + B}$$

In kinetic resolution experiments using hydrolytic enzymes it is also desirable to relate the extent of conversion( $c$ ) and the enantiomeric excess of the product fraction  $ee(P)$  to various values of  $E$ .

$$\frac{\ln[1 - c(1 + ee(P))]}{\ln[1 - c(1 - ee(P))]} = E$$

where,

$$c = 1 - \frac{A + B}{A_0 + B_0}$$

$$ee(P) = \frac{P - Q}{P + Q}$$

## 1.8. THE PROPOSED WORK: THREE PHASE ENZYMATICAL REACTOR

In this work, a novel three phase enzymatic reactor for continuous optical resolution of racemic mixtures of chiral alcohols is described. The strategy involves enzyme mediated enantioselective transport of only one enantiomer of the racemic mixture. The three phases consist of two aqueous phases and one organic phase. The aqueous phases are physically separated from each other by an impermeable glass partition. The organic phase is in contact with the two aqueous phases. The enzyme present in the first aqueous phase enantioselectively acetylates the hydrophilic racemic alcohol, essentially at interface 1. The acetylated product is extracted into the organic phase. At interface 2, the enzyme in the second aqueous phase hydrolyses the acetylated alcohol. At the end of the reaction, one isomer of the racemic mixture is obtained in the second aqueous phase, while the other isomer remains in the first aqueous phase. The performance of the "three phase enzymatic reactor" is demonstrated on a laboratory scale.

## CHAPTER 2: EXPERIMENTAL SECTION

### 2.1. GENERAL

All experiments were performed in oven dry glass apparatus .Reaction mixtures were magnetically stirred unless otherwise specified . All reactions were performed at 20° C.

Thin layer chromatography (TLC) was performed on prepared thin layers of E.Merck Silica gel-G and Acme Silica gel-G (Bombay,India) on microscopic slides.The slides were dried at room temperature prior to use.Visualization of spots was effected by ultraviolet illumination or exposure to iodine vapours.

The  $[\alpha]_D^{20}$  value of optically active compounds was recorded on Autopol II automatic polarimeter at the wavelength of the sodium D-line (589nm)and at ambient temperature .

## **2.2. MATERIALS**

The enzyme porcine pancreatic lipase (PPL) was commercially available from Fluka. Pig Liver Acetone Powder (PLAP) was freshly prepared in the laboratory.

### **2.2.1. Preparation Of Pig Liver Acetone Powder (PLAP)**

The procedure for preparation of PLAP is given in literature (Adachi et al, 1986). Freshly purchased pig liver (500g) was homogenized in chilled acetone (2l) using kitchen juicer. The brown mass obtained after filtration was air dried at room temperature and powdered using grinder. Fibrous material was removed by sieving to furnish 100g of PLAP as fine powder. This powder can be stored for 2,3 months in refrigerator without any significant loss of activity.

### **2.2.2. Preparation of Phosphate Buffer**

Solution A is prepared by dissolving 3.12g of  $\text{KH}_2\text{PO}_4$  in 100 ml of distilled water  
Solution B is prepared by dissolving 2.84g  $\text{K}_2\text{HPO}_4$  in 100 ml distilled water .Both solutions are of molarity 0.2M. To make buffer of pH 8.0, 5.3 ml solution A and 94.7 ml solution B are mixed together .

### 2.2.3. Substrates

The substrates lactic acid (2-hydroxy propionic acid), mandelic acid (1-phenyl, 1-hydroxy ethanoic acid) and vinyl acetate used for acetylation were obtained from commercial sources. The diols, 1,2-propane diol and 1-phenyl, 1,2-ethane diol were then prepared using these substrates.

### 2.2.4. Esterification of lactic acid

A mixture of 2g of lactic acid, anhydrous ethanol (10ml), anhydrous benzene (50ml) and catalytic amount of p-toluene sulphonic acid (TSOH) was subjected to Dean Stark removal of benzene water azeotrope. On completion of the reaction (TLC monitoring), the reaction mixture was neutralised with saturated  $\text{NaHCO}_3$  solution (15ml) and extracted with  $\text{Et}_2\text{O}$  (3\*25 ml) in the usual manner. The combined ethereal layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Concentration of the solvent in a rotary evaporator afforded ethyl ester in high purity.

### 2.2.5. Preparation of 1,2 - propane diol

The ethyl ester (1g, 8.47mmol) dissolved in anhydrous THF (5ml) was added gradually to the suspension of  $\text{LiAlH}_4$  (0.8g, 21.2mmol) in anhydrous THF (10ml) under argon atmosphere. The reaction mixture was refluxed for 6 hours. On completion of the reaction (TLC monitoring), the excess of  $\text{LiAlH}_4$  was destroyed by successive addition of ethyl acetate and water and the reaction mixture was filtered through a sintered funnel. The residue was washed thoroughly with ethyl acetate (50ml) and the organic layer was stored

over anhydrous  $\text{Na}_2\text{SO}_4$ . Concentration of the solvent on a rotary evaporator afforded the crude product (diol) which was purified by column chromatography.

[eluent:petroleun ether-ethyl acetate /1:1]

#### **2.2.6. Esterification of Mandelic acid**

A mixture of 2g of mandelic acid ,anhydrous ethanol (10ml),anhydrous benzene (50ml) and catalytic amount of p-tolune sulphonic acid (TSOH) was subjected to Dean Stark removal of benzene water azeotrope.On completion of the reaction (TLC monitoring), the reaction mixture was neutralised with saturated  $\text{NaHCO}_3$  solution (15ml) and extracted with  $\text{Et}_2\text{O}$  (3\*25 ml) in the usual manner . The combined ethereal layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Concentration of the solvent in a rotary evaporator afforded ethyl ester in high purity.

#### **2.2.7. Preparation of 1-phenyl, 1,2- ethane diol**

The ethyl ester (1 g , 5.56 mmol) dissolved in anhydrous THF (5ml) was added gradually to the suspension of  $\text{LiAlH}_4$  ( .527 g, 13.89 mmol) in anhydrous THF (10ml) under argon atmosphere .The reaction mixture was refluxed for 6 hours.On completion of the reaction (TLC monitoring), the excess of  $\text{LiAlH}_4$  was destroyed by successive addition of ethyl acetate and water and the reaction mixture was filtered through a sintered funnel . The residue was washed thoroughly with ethyl acetate (50ml) and the organic layer was stored over anhydrous  $\text{Na}_2\text{SO}_4$ . Concentration of the solvent on a rotary evaporator afforded the crude product (diol) which was purified by column chromatography.

[eluent:petroleun ether-ethyl acetate /1:1]

## **2.3. BIPHASIC REACTIONS**

### **2.3.1. Reaction using PPL**

To a biphasic mixture of 10ml buffer and 10ml hexane, lactic acid (600mg, 6.67 mmol) and vinyl acetate (4g, 46.7 mmol) were added. PPL was added in catalytic amount (15mg). The entire mixture was stirred to form a uniform suspension. The reaction was continuously monitored by TLC and stopped after 48 hrs, on obtaining a distinct spot of the expected product, i.e. acetylated lactic acid.

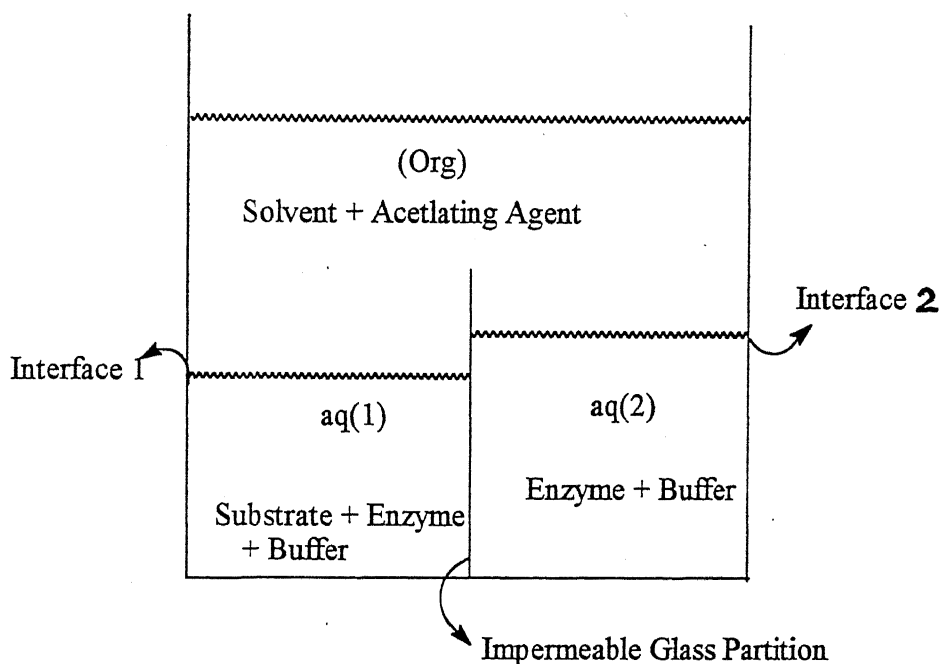
### **2.3.2. Reaction using PLAP**

To a biphasic mixture of 10ml buffer and 10ml hexane, lactic acid (600 mg, 6.67 mmol) and vinyl acetate (4g, 46.7mmol) were added. PLAP was added in small amount (15mg). The entire mixture was stirred to form a uniform suspension. The reaction was continuously monitored by TLC and stopped after 90 hrs, on obtaining a distinct spot of the expected product i.e. acetylated lactic acid.

## **2.4. THREE PHASE REACTIONS**

The three phase enzymatic chiral resolution reactions were carried out in a glass assembly as shown in the schematic diagram (Figure 1).





**Figure 1 : Schematic of Three Phase Enzymatic Reactor**

The glass assembly consisted of two concentric glass cylinders. The outer cylinder was of 5cm diameter and 7cm height. The inner cylinder was 2.5 cm in diameter and 3.5cm high. The aqueous phase in the inner cylinder was called aq(1) and that in the outer cylinder was called aq(2). The organic phase (org) formed a continuous layer on top of the aqueous phases, thus facilitating transport from aq(1) to aq(2) through the organic phase. Thus in the current assembly the interfacial area at interface 1 =  $4.9 \text{ cm}^2$  and at Interface 2 =  $14.74 \text{ cm}^2$ . Phosphate buffer of pH 8.0 was used in the aqueous phases and a water immiscible organic solvent (hexane in the present work) formed the organic phase.

#### 2.4.1. General Procedure for Three Phase Reactions

The racemic mixture of the alcohol(3-6 mmol) and the enzyme(15-100mg) were dissolved in 10ml of the buffer solution in aqueous phase aq(1). The second aqueous phase aq(2) contained 15-100mg enzyme dissolved in 30ml buffer solution. The organic phase contained Vinyl acetate(4-5 equivalents of the substrate) dissolved in 30ml organic solvent. Both the aqueous phases were continuously and separately stirred. The progress of reactions was continuously monitored by TLC and also by measuring the optical rotation of the organic phase. A 2ml aliquote was removed from the organic phase at differnt instants of time to measure the optical rotation. The same aliquote was added to the organic phase immediately after measurement of optical rotation.

After stopping the reaction, the three phases were separated. The aqueous phases were separately extracted with ethyl acetate, dried over  $\text{Na}_2\text{SO}_4$  and concentrated on rotory evaporator. The weight of residue left after drying the ethyl acetate layer corresponding to each aqueous phase was noted to calculate the percentage of transport. The optical rotation of the compound in each aqueous phase was also measured to calculate the enantiomeric excess.

#### 2.4.2. Control Reaction

Corresponding to each of the “three phase enzymatic reaction” , a control reaction was carried out. The procedure was the same as described above, except that no enzyme was used in either of the aqueous phases. The organic phase was continuously monitored by

measuring the optical rotation. After stopping the reaction, the aqueous phases were separately extracted with ethyl acetate. The ethyl acetate layer was then dried over  $\text{Na}_2\text{SO}_4$ , concentrated on rotory evaporator and checked for any transport of material.

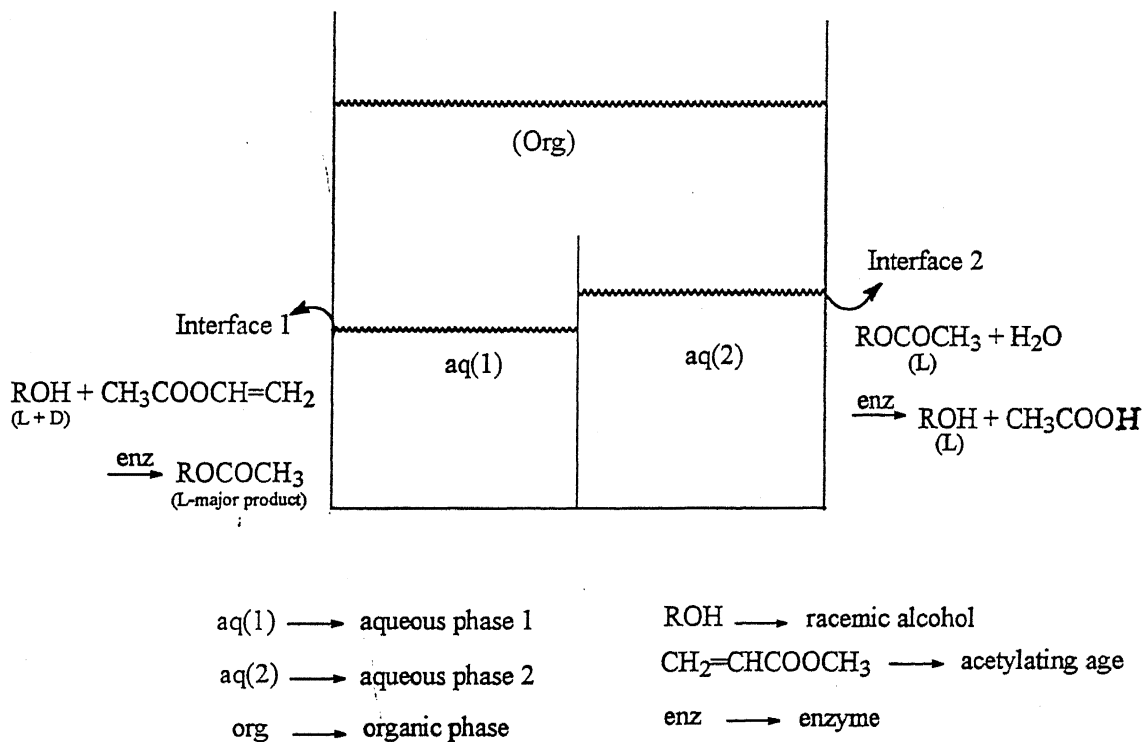
## CHAPTER 3: RESULTS AND DISCUSSION

### 3.1. Objectives

The application of lipases in kinetic resolution of racemic mixtures of alcohols or acids is well known (de Zoete et al, 1994; Klibanov, 1990). Monophasic and biphasic systems for such lipase catalysed chiral resolutions have been reported (Abramowicz and Keese, 1987). In this work, a novel "Three Phase Enzymatic Reactor" for continuous resolution of racemic mixtures of hydroxy compounds has been developed. The schematic of the laboratory scale reactor devised for this purpose is shown in fig 2. The two aqueous phases aq(1) and aq(2), were separated by an impermeable glass partition. However, both the aqueous phases were in contact with the organic phase ,org.

The goal was to start with a racemic mixture (ie. 50:50 mixture of L and D isomers) of an alcohol in aq(1) and at the end of separation process obtain one of the isomers (eg. L isomer ) in aq(2) and the other isomer in aq(1). To achieve this, we employed one of nature's best strategies, facilitated transport. In other words , the transport of one of the enantiomers from aq(1) to aq(2) through the organic phase was facilitated by enzymes. Essentially, at interface 1, lipase catalysed enantioselective acetylation of the alcohol followed by extraction into the organic phase facilitated enantioselective transport across interface 1, into the organic phase. At interface 2, the acetylated product was hydrolysed

and the alcohol produced was transported across interface 2 into the aqueous phase aq(2). Both the aqueous phases should have suitable hydrolytic enzymes. The racemic alcohol should be soluble only in water and the acetylated product should be soluble in the organic solvent.



**Figure 2 : Schematic of Three Phase Reactor Showing reactions at the Interface**

### 3.2. SELECTION OF ENZYME

In this work, we have used lipases, which come under the general classification of hydrolytic enzymes. The selection of lipases were based on their ability to catalyse enantioselective transesterification and hydrolysis and also on their availability. Lipase

from porcine pancreas(PPL) was selected due to its low cost, high stability and substrate tolerance. In one of the runs, freshly prepared pig liver acetone powder (PLAP) was made use of.

### 3.3. SELECTION OF SUBSTRATES

The racemic alcohols would have to fulfill certain criteria in order to be suitable candidates for carrying out chiral resolution via this approach. First of all, the alcohol should be reasonably soluble in water but should be insoluble in the organic solvent. Solubility of the alcohol in organic phase can lead to passive and in turn non-enantioselective transport from aq(1) to aq(2) across the organic phase. The acetylated product, however, should have reasonable solubility in organic phase. Also, the alcohol should be an acceptable substrate for the lipase used. Biphasic reactions were conducted for lactic acid to analyse the reaction conditions of the lipase, time of reaction, product formation and behaviour of product. Similar reactions using 1-phenyl, 1, 2-ethane diol and various lipases were carried out and good results were obtained (Roy,1997). A large number of enzymatic transformations using propane diol and various lipases have been reported in literature (Klibanov, 1990 ; Chen et al, 1989; de Zoete et al, 1994). Studying these reactions helped in planning and carrying out the "three phase reactions" in an efficient manner.

### 3.4. SELECTION OF THE PHASES

A water immiscible organic solvent was selected in which the substrate was insoluble but the acetylated product was soluble. After trying out several solvents, hexane was selected as the organic phase. Other solvents like ether were found to cause significant amount of passive transport due to the increased solubility of the substrate. A phosphate buffer of pH 8.0 was used as aqueous phase to keep the enzyme activity optimum. Vinyl acetate was chosen as the acetylating agent essentially because of its ability to prevent the reverse reaction by the formation of acetaldehyde.

### 3.5. BIPHASIC REACTIONS

Biphasic reactions were carried out using lactic acid and two different enzymes, PPL and PLAP. Table 2 gives the reaction results.

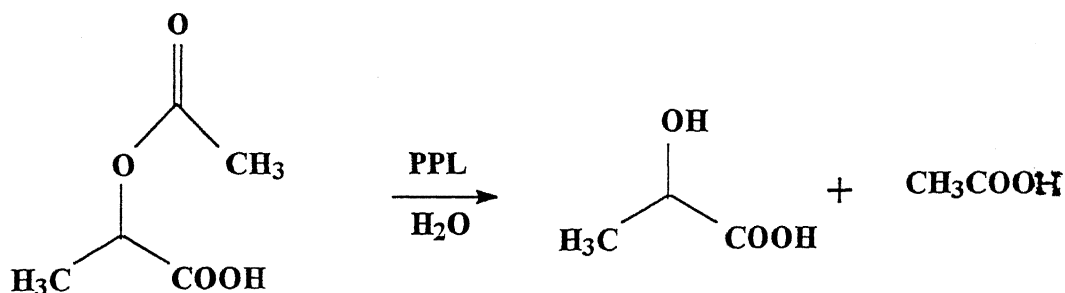
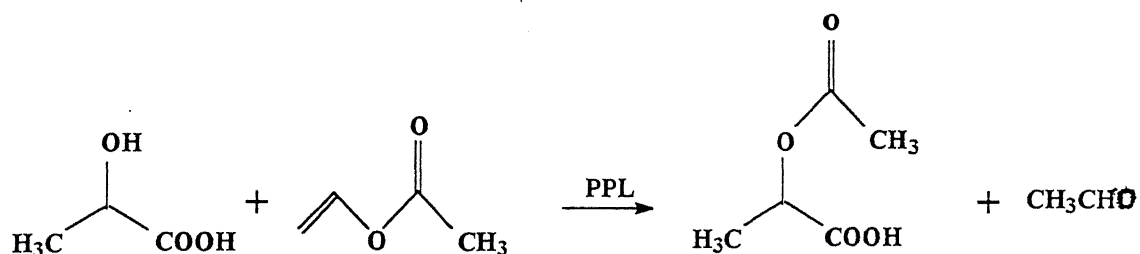
**Table 2:**Results of biphasic reactions using lactic acid.

Enzyme	Amount	Solvent	Acyl Donor	Temp ° C	Time (h)	$[\alpha]_D$
PPL	15 mg	Hexane	VA	20	48	2°
PLAP	15 mg	Hexane	VA	20	84	2°

The reaction using PPL was much faster compared to the one using PLAP. The product formed in both the reactions were found to be the same as they showed same degree of optical rotation. Based on this result, PPL was chosen for acetylation in the three phase reactions.

### 3.6. THREE PHASE REACTIONS

#### 3.6.1. Run 1: Lactic acid + Vinyl acetate using PPL in aq(1) and PPL in aq(2)



The reaction was carried out as described in the experimental section. Table 3 shows the variation of the optical rotation of the organic phase with time and the same is plotted in fig 3.



**Table 3: Three Phase Reaction Run 1.**

Lactic Acid + Vinyl Acetate using PPL

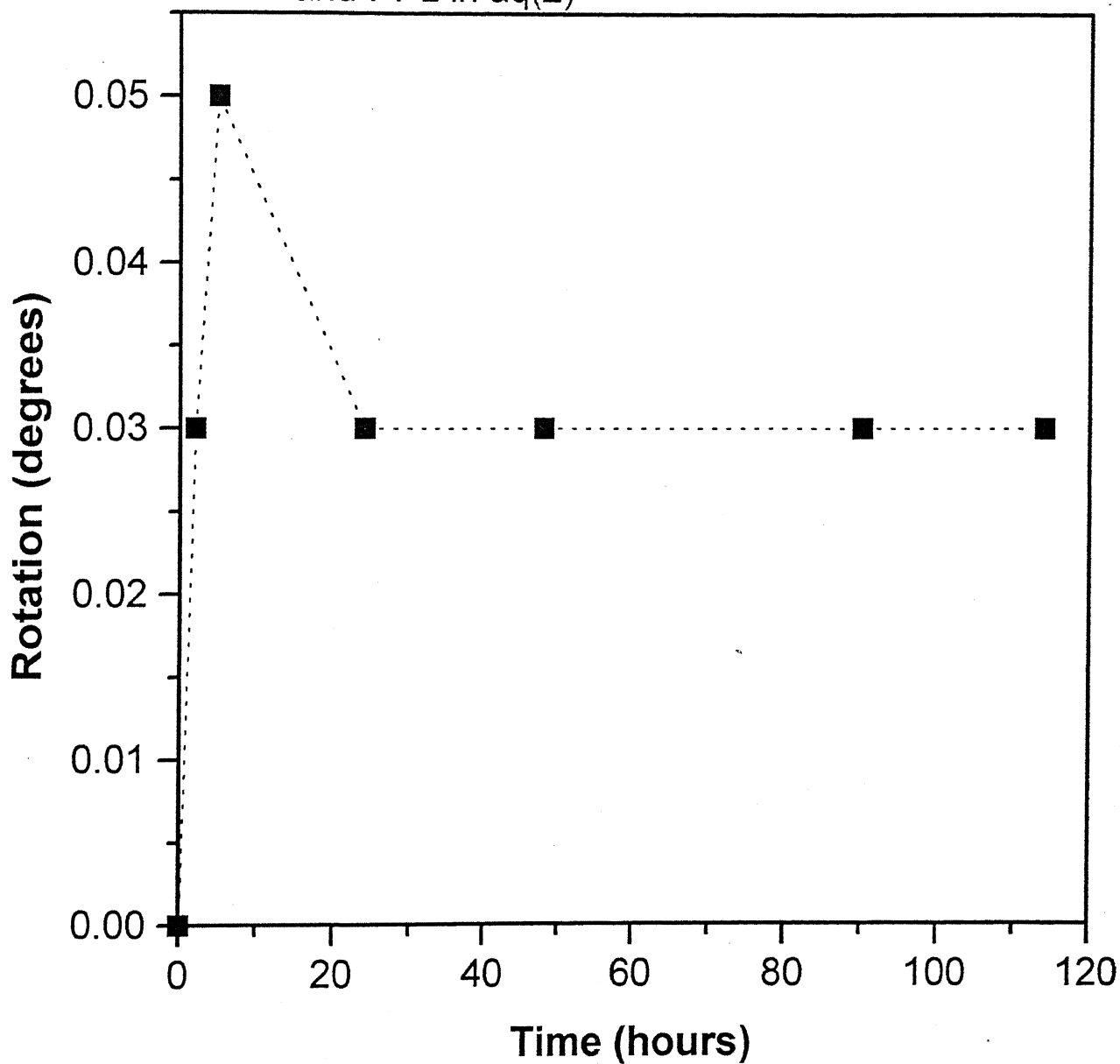
Time (h)	Rotation
0	+0.00
2	+0.03
5	+0.05
24	+0.03
48	+0.03
90	+0.03
114	+0.03

Initially, the organic phase showed zero rotation. As the reaction progressed, the rotation increased in the positive direction and then decreased to remain constant at +0.03. The increase in the value of rotation was the indication of the formation of acetylated lactic acid and its transfer into the organic phase. The later decrease in rotation value indicated that acetylated lactic acid was getting hydrolysed, which was later confirmed by the presence of lactic acid in the second aqueous phase aq(2).

The reaction was stopped after 114 hours since the rotation value remained constant after 24 hours. This could be due to the reduction in the rate of acetylation and the slow rate of

hydrolysis. The reaction was stopped to prevent the slow reacting enantiomer from getting acetylated, which would affect the enantiomeric excess. The enantiomeric excess couldn't

Fig.3: Run1- Lactic acid + Vinyl acetate using PPL in aq(1) and PPL in aq(2)



be calculated in this case because the compounds from both aqueous phases gave positive rotations which couldn't be compared with known values. This could be due to the presence of acetylated lactic acid in all the three phases which had a dominating effect on the optical rotation. Acetylated lactic acid has significant solubility in the aqueous phase .

### 3.6.2.Run 2: Lactic acid + Vinyl acetate using PPL in aq(1) and PLAP in aq(2).

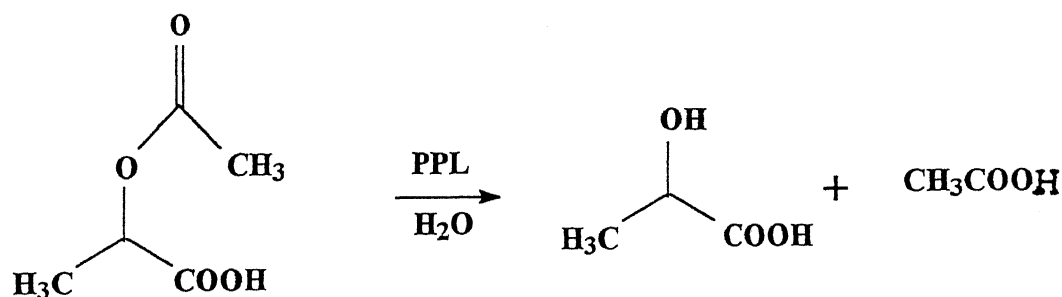
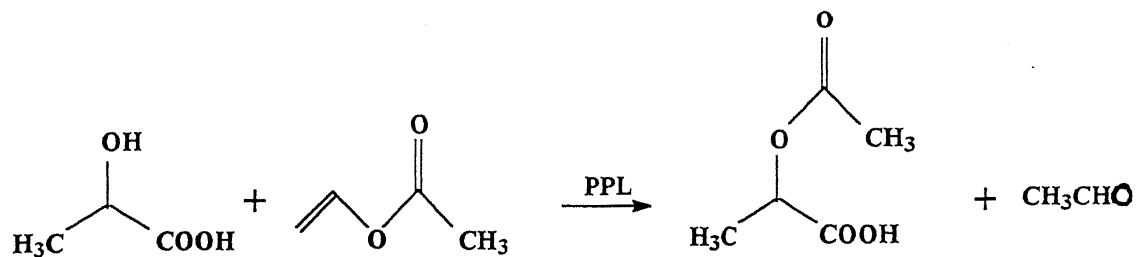


Table 4 shows the variation in the rotation of the organic phase with time and Fig 4 shows the plot for the same.

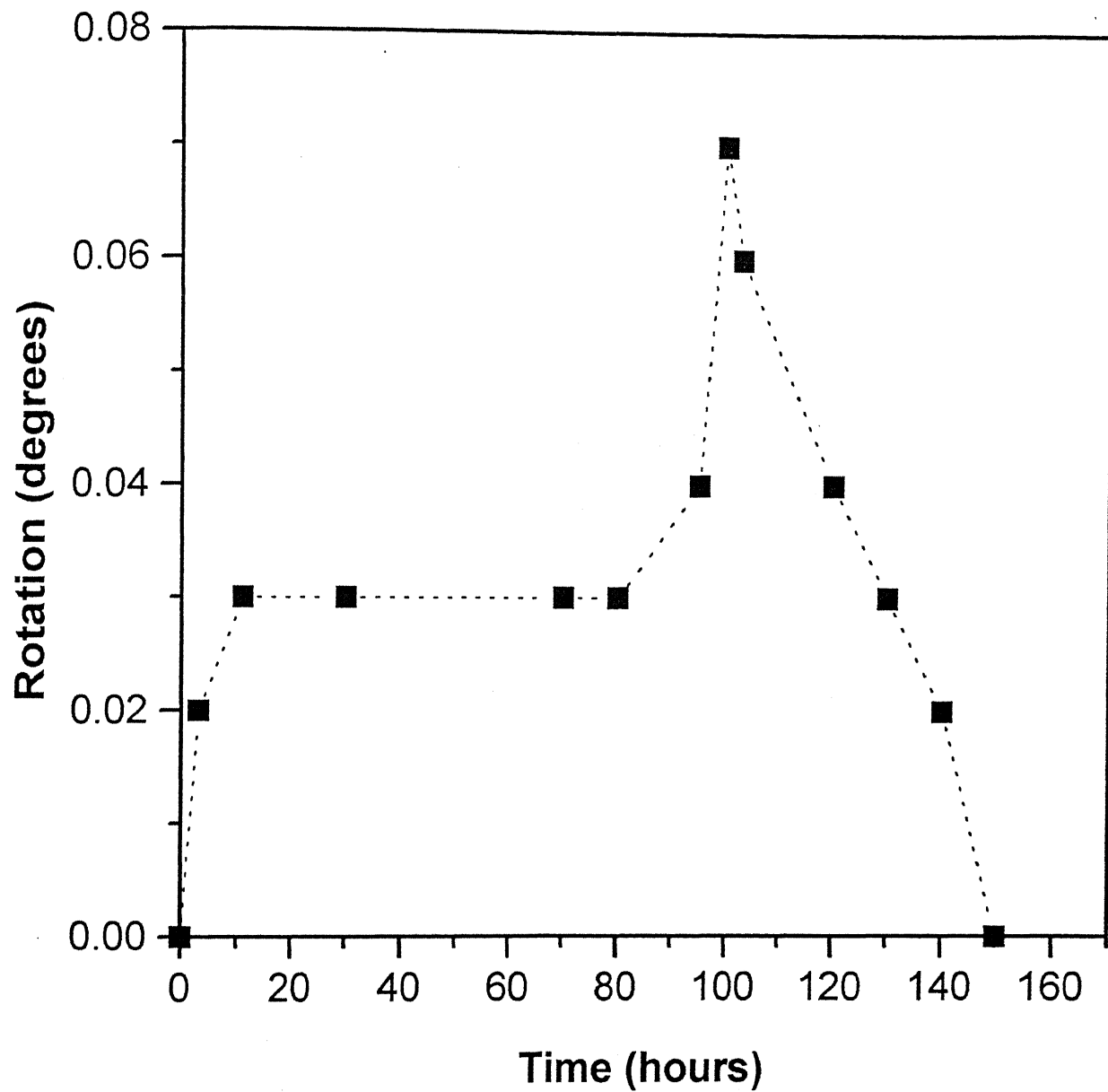
**Table 4: Three Phase Reaction Run 2.**

Lactic Acid + Vinyl Acetate using PPL in aqueous phase 1 and PLAP in aqueous phase 2.

Time (h)	Rotation
0	+0.00
3	+0.02
11	+0.03
30	+0.03
70	+0.03
80	+0.03
95	+0.04
100	+0.07
103	+0.06
120	+0.04
130	+0.03
140	+0.02
150	+0.00

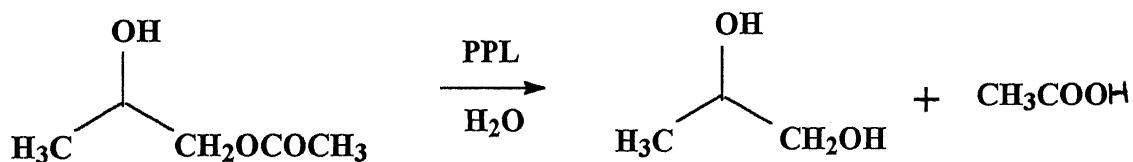
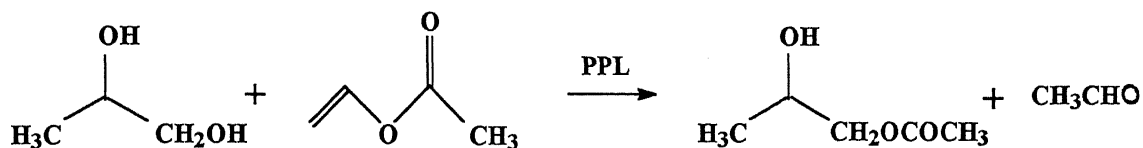
The optical rotation increased gradually from 0.0 to a value of +0.07 from where it declined to 0.0. The decrease in rotation began only after 100 hours because of the slow

Fig.4: Run2- Lactic acid + Vinyl acetate using PPL in aq(1) and PLAP in aq(2)



hydrolysis by PLAP in the aqueous phase aq(2). The decline of the rotation to 0.0 could have been due to the complete hydrolysis of the acetylated lactic acid. But the presence of the acetylated product in the organic phase at the end of the reaction(after separation and concentration) contradicted this. Therefore decrease in rotation could be attributed to the acetylation of the slow reacting enantiomer. In this case also, enantiomeric excess could not be determined due to reasons given in Run 1.

### 3.6.3.Run 3: 1,2-Propane diol + Vinyl acetate using PPL in aq(1) and PPL in aq(2).



Monoacetate formation during enzymatic acetylation of propane diol using lipases have been reported in literature (Klibanov, 1990).

Table 5 shows the variation in rotation of the organic phase with time and Fig 5 shows the plot for the same.

The pattern of variation in rotation was found to be nearly the same as for Run 2. PPL was used for the hydrolysis reaction in aqueous phase aq(2). Since the rotation remained constant for about 72 hours, it can be inferred that the rate of acetylation and hydrolysis

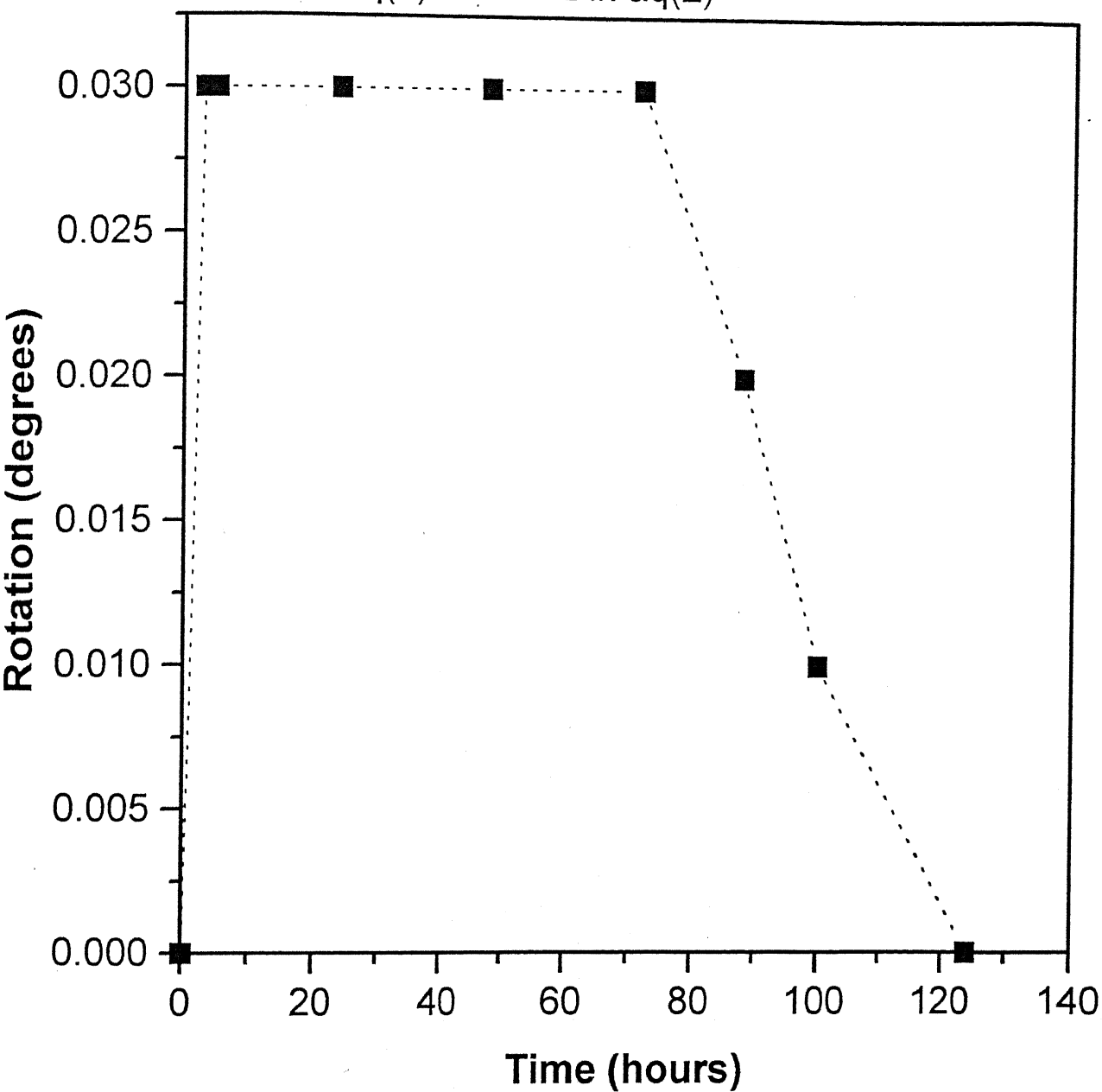
were almost equal. The subsequent decrease in rotation value could be due to the acetylation of the slow reacting enantiomer. The enantiomeric excess could not be determined in this case due to the unavailability of known values .

**Table 5:** Three Phase Reaction Run 3.

1,2- Propane Diol + Vinyl Acetate Using PPL.

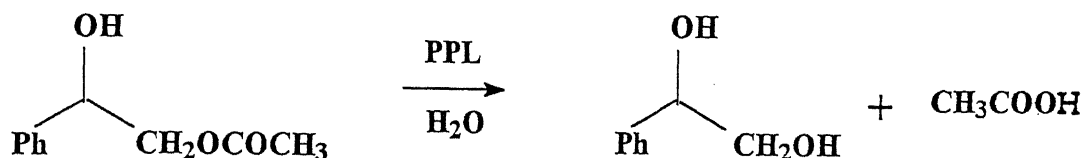
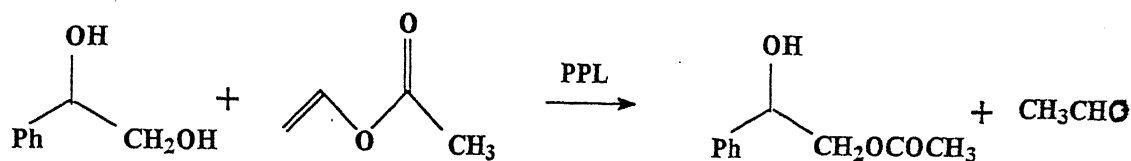
Time (h)	Rotation
0	+0.00
3	+0.03
5	+0.03
24	+0.03
48	+0.03
72	+0.03
88	+0.02
100	+0.01
124	+0.00

Fig.5: Run3- 1,2-Propane diol + Vinyl acetate using PPL in aq(1) and PPL in aq(2)





3.6.4. Run 4: 1-Phenyl,1,2-ethane diol + Vinyl acetate using PPL in aq(1) and PPL in aq(2).



NMR data has shown that the primary hydroxy group gets acetylated by the action of the lipase (Roy,1997).

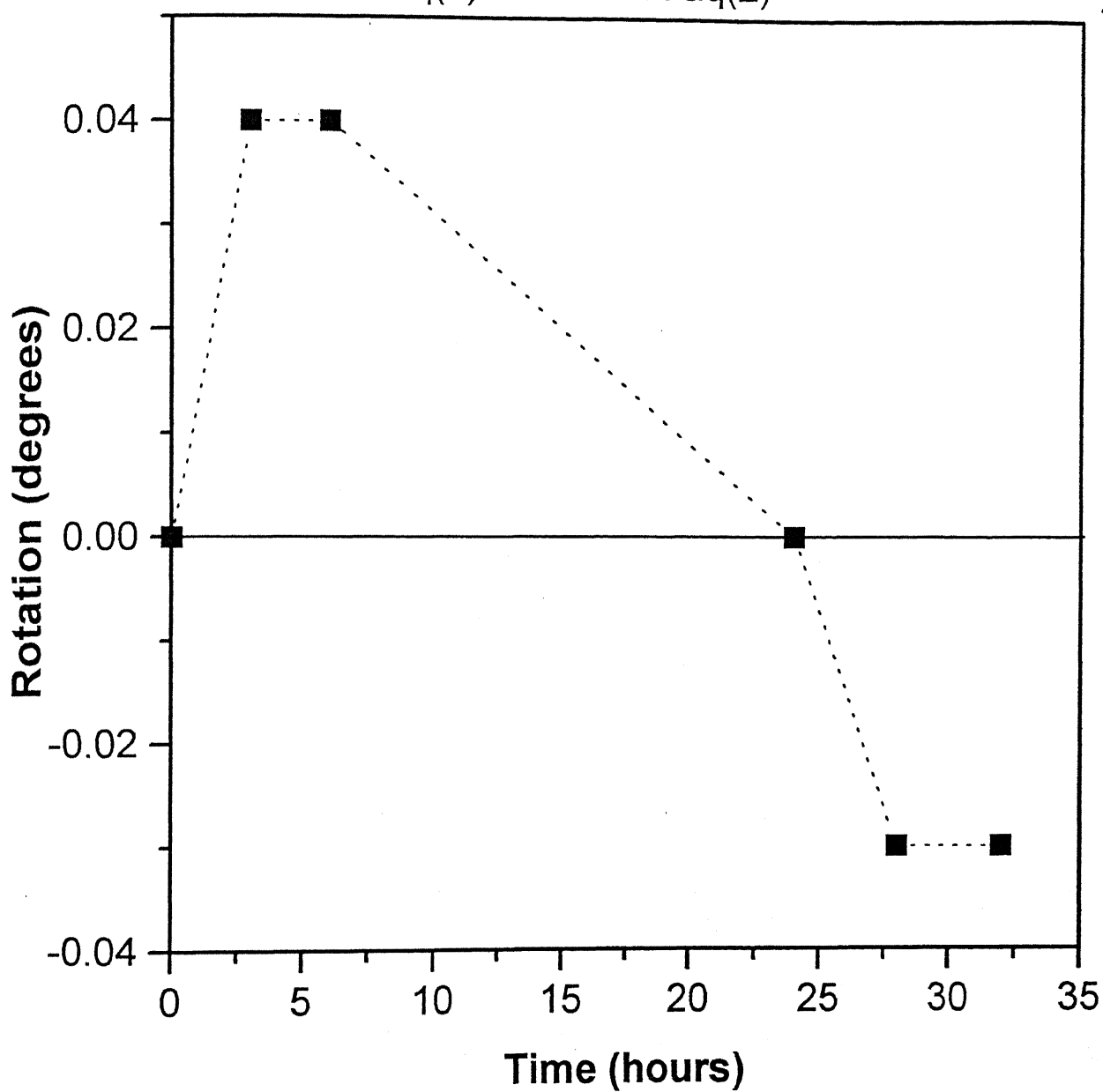
Table 6 gives the variation in rotation of the organic phase with time and Fig 6 shows the plot for the same.

**Table 6 :Three Phase Reaction Run 4.**

1 - Phenyl, 1,2- Ethane Diol + Vinyl Acetate using PPL.

Time (h)	Rotation
0	+0.00
3	+0.04
6	+0.04
24	+0.00
28	-0.03
32	-0.03

Fig.6: Run4- 1-Phenyl,1,2-Ethane diol + Vinyl acetate using PPL in aq(1) and PPL in aq(2)



As shown in Fig 6, this run gave an unusual pattern of optical rotation of the organic phase, in comparison with the other runs. Negative rotation was observed only in this run. The rotation increased from 0.0 to +0.04, then decreased to 0.0 and declined further showing negative rotation of -0.03. This unusual behaviour could be due to the presence of a second enzyme in crude PPL which was selective towards the (-)-enantiomer and which started dominating the later stage of the reaction.

The enantiomeric excess was determined by comparison of the observed specific rotation with the known value from literature. The reported value was  $+39^{\circ}(\text{c3,EtOH})$ . The observed rotation for aq(1) was  $+2^{\circ}(\text{c3,EtOH})$  and that for aq(2) was  $-3^{\circ}(\text{c3,EtOH})$ . The enantiomeric excesses (ee) were calculated as given in the introduction and were found to be 5.12% for aq(1) and 7.69% for aq(2). Low values of ee were obtained because both the enantiomers had undergone the reaction by the action of two different enzymes.

### 3.6.5. Control Reactions

Control reactions without enzymes were done for each of the three substrates, as described in the experimental section. The organic phase did not show any optical rotation during the entire period the reaction was run. The absence of any residue in the aqueous phase aq(2) confirmed that there was no passive transport of the substrate from aqueous phase aq(1) to aqueous phase aq(2).

### 3.6.6. Overall Results of the Three Phase reactions

The overall results of the three phase reactions are tabulated in table 7.

Table 7:Results of Three Phase Reactions.

Run	Substrate	Enz aq(1)	Enz aq(2)	Time (hrs)	% active <sup>a</sup> Transport	% passive <sup>b</sup> Transpo rt	ee <sup>c</sup> aq(1)	ee <sup>d</sup> aq(2)
1	Lactic cid	PPL	PPL	114	5	0	nd <sup>e</sup>	nd <sup>e</sup>
2	Lactic acid	PPL	PLAP	150	3	0	nd <sup>e</sup>	nd <sup>e</sup>
3	1,2- propane diol	PPL	PPL	124	11	0	nd <sup>e</sup>	nd <sup>e</sup>
4	1-phenyl, 1,2-ethane diol	PPL	PPL	32	14	0	5.12%	7.69%

a: % active transport :- Transport of material from aq(1) to aq(2) due to the action of enzymes.

b: % passive transport :- Transport of material from aq(1) to aq(2) in the absence of enzymes.

c: ee aq(1) :- Enantiomeric excess of substrate in aqueous phase 1.

d: ee aq(2) :- Enantiomeric excess of substrate in aqueous phase 2.

e: nd :- Not determined

The "three phase enzymatic reactions", transport of material from aqueous phase aq(1) to aqueous phase aq(2) was only by the action of enzymes. Passive transport was not observed in any of the runs. The percentage of active transport for lactic acid was less as compared to the other two substrates. The highest transport that was obtained in the case of 1-phenyl, 1,2-ethane diol could be due to the increased rate of enzymatic transformation. In Run 2, where PLAP was used for hydrolysis, percentage transport was very small due to the slow rate of hydrolysis.

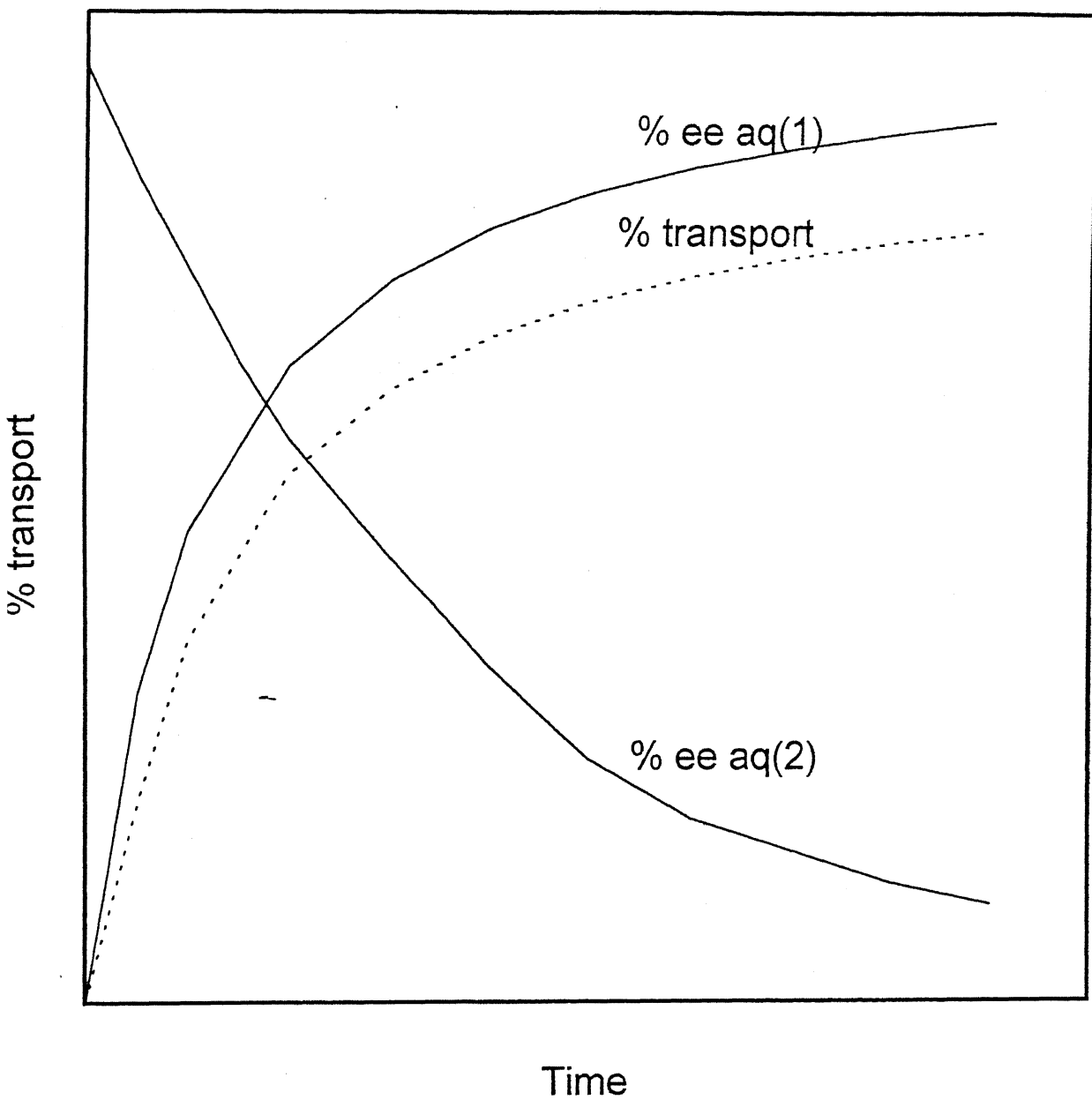
Enantiomeric excess (ee) could be determined for Run 4 only and it was found to be approximately 7%. This could be because both the enantiomers got transferred by action of two different enzymes present in crude PPL, without significant difference in the rate of reactions.

### **3.7. FUTURE WORK : OVERALL KINETICS OF THE "THREE PHASE ENZYMATIC REACTOR"**

The chiral resolution strategy used in the "three phase enzymatic reactor" is a kinetic process. The percentage transport is expected to increase from the initial zero percentage to a maximum of 50 percent with time, depending on the rate of reactions and rate of mass transfer at both the interfaces. The rate of reaction depends on the area of the reactor, rates of mass transfer between the phases, activity of the enzymes and so on.

The enantiomeric excesses (ee) of the two aqueous phases aq(1) and aq(2) can be calculated at different instants of time by measuring the optical rotations of the two phases. The expected values of ee are : for aqueous phase aq(1) ee will be zero initially which will increase as the reaction progresses, due to the selective reaction by the enzyme. The ee of aqueous phase aq(2) will show maximum value initially and will decrease with time as the slow reacting enantiomer starts getting acetylated in the later stages of the reaction. Figure 7 shows the expected plot for the kinetics of three phase enzymatic reactor. The reactor is expected to give optimum ee in both aqueous phases and a substantial amount of facilitated transport from aq(1) to aq(2) across the organic phase.

Fig 7: Expected plot for kinetics of three phase enzymatic reactor



## CHAPTER 4 :CONCLUSIONS

In this work, continuous optical resolution of racemic alcohols by a "three phase enzymatic reactor" using lipases was demonstrated on a laboratory scale. The percentage of facilitated as well as passive transport were measured for each substrate. The enantiomeric excesses (ee) of both aqueous phases aq(1) and aq(2) were calculated for 1-phenyl,1,2-ethane diol.

The percentage of facilitated transport varied for each substrate. This variation in the percentage of transport could be attributed to the differences in the activity of the enzymes towards each substrate. Passive transport was not observed in any of the three phase reactions. The enantiomeric excesses(ee) measured for 1-phenyl,1,2-ethane diol were not significantly high. It was hypothesized that small value of ee was due to the presence of other enzymes in crude PPL, whose selectivity differed from that of PPL.

On the basis of the above results, it can be said that the principle of enantioselective enzymatic transformation followed by extraction across liquid- liquid interfaces is an effective method for optical resolution of racemic compounds. With the selection of proper solvent and enzyme having right stereoselectivity, the "three phase enzymatic reactor" can be used for continuous optical resolution.



The performance and overall kinetics of the "three phase reactor" are affected by factors like interfacial area, total volume, rates of mass transfer between phases etc. Further kinetic studies are required to optimise the "three phase enzymatic reactor". The chiral resolution strategy of enantioselective transformation followed by extraction can be extended to other enzymatic systems such as alcohol dehydrogenase. Such three phase enzymatic reactors can be scaled up and applied for chiral resolution on an industrial scale. Industries like pharmaceuticals, agrochemicals, food additives etc. where enantiomeric compounds are of much importance, can make use of the "three phase enzymatic reactor", for resolution of racemic compounds.

## REFERENCES

- Abramowicz, D.A., Keese, C.R. (1989) "Enzymatic Transesterifications of Carbonates in Water Restricted Environments", *Biotechnology and Bioengineering*, **33**, 149-156.
- Adachi, K., Kobayashi, S., Ohno, M. (1986), "Chiral Synthons by Enantioselective Hydrolysis of Meso-diesters with PLE: Substrate-Stereoselectivity Relationships", *Chimia*, **40**, 311-316.
- Ching-Shih Chen, Sih, C.J. (1989), "General Aspects and Optimisation of Enantioselective Biocatalysis in Organic Solvents: The Use of Lipases", *Angew. Chem. Int. Ed. Engl.*, **28**, 695-707.
- Ching-Shih Chen, Fujimoto, Y., Girdaukas, G., Sih, C.J. (1982), "Quantitative Analysis of Biochemical Kinetic Resolutions of Enantiomers", *J. Am. Chem. Soc.*, **104**, 7294-7299.
- Cygler, M., Grochulski, P., Kazlauskas, R., Schrag, J.D., Bonthillier, F., Rubin, B., Serrege, A.N., Gupta, A.K. (1994), "A Structural Basis for the Chiral Preferences of Lipases", *J. Am. Chem. Soc.*, **116**, 3180-3186.
- Dakin, H. D. (1903), *Proc. Chem. Soc. London*, **19**, 161.
- Gandhi, N.N., Sawant, S.B., Joshi, J.B., (1995), "Specificity of a Lipase in Ester Synthesis: Effect of Alcohol", *Biotechnology Progress*, **11**, 282-287.
- Jones, J.B., Sih, C.J., Perlmann, D. (1976), *Tech. Chem. (Prague)*, **10**, 107.
- Klibanov, A.M. (1990), "Asymmetric Transformations Catalysed by Enzymes in Organic Solvents", *Acc. Chem. Res.*, **23**, 114-120.

Koskinen, A.(1993), "Asymmetric Synthesis of Natural Products", *John Wiley and Sons Ltd., England*.

Lopez, J.L., Matson, S.L. (1997), "A Multiphase/Extractive Enzyme Membrane Reactor For Production of Diltiazem Chiral Intermediate", *J. Memb. Sci.*, **125**, 189-211.

Martin, V.S., Woodard, S.S, Katsuki, T., Yamada, Y., Ikeda, M., Sharpless, B. K. (1981), "Kinetic Resolution of Racemic Allylic Alcohols by Enantioselective Epoxidation: A Route to substances of Absolute Enantiomeric Purity", *J. Am. Chem. Soc.*, **103**, 6237-6240.

Morrison, R. T., Boyd, R. N. (1995), "Organic Chemistry (6<sup>th</sup> edition)", *Prentice-Hall of India Pvt. Ltd., New Delhi*.

Roy, S., (1997), "Lipase Catalysed Enantioselective Transformations in Organic Media", *M.Tech Thesis, IIT, Kanpur.*,

Schoffers, E., Golebiowski, A., Johnson, C.R.(1996), "Enantioselective Synthesis Through Enzymatic Asymmetrization ", *Tetrahedron*, **52**, 3769-3826.

Theil, F. (1995), "Lipase Supported Synthesis of Biologically Active Compounds", *Chem. Rev.*, **95**, 2203-2227.

Uppenberg, J., Ohrner, N., Norin, M., Hult, K., Kleywegt, G.J., Patkar, S., Wargen, V., Anthonsen, T., Jones, A. T. (1995), "Crystallographic and Molecular Modelling Studies of Lipase B from *Candida Antarctica* Reveal a Stereospecificity Pocket for Secondary Alcohols", *Biochemistry*, **34**, 16838-16851.

Wescott, C.R., Klibanov, A.M. (1994), "The Solvent Dependence of Enzyme Specificity", *Biochimica et Biophysica Acta*, **1206**, 1-9.

Wang, Y., Lalonde, J.J, Momongan, M., Bergbreiter, D.E., Wong, C.H. (1988), "Lipase Catalysed Irreversible Transesterifications using Enol Esters as Acylating Reagents: Preparative Enantio and Regioselective Synthesis of Alcohols, Glycerol Derivatives, Sugars and Organo Metallics", *J. Am. Chem. Soc.*, **110**, 7200-7205.

Wong, C.H., Whitesides, G.M. (1994), "Enzymes in Synthetic Organic Chemistry", *Elsevier Science Ltd., Oxford, U.K.*

de Zoete, M.C., van Rantwijk, F., Sheldon, R.A. (1994), "Lipase Catalysed Transformations with Unnatural Acyl Acceptors", *Catalysis Today*, **22**, 563-590.

